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Selected Antimicrobial Essential Oils Eradicate *Pseudomonas* spp. and *Staphylococcus aureus* Biofilms

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Biofilms are difficult to eliminate with standard antimicrobial treatments due to their high antibiotic resistance relative to free-living cells. Here, we show that selected antimicrobial essential oils can eradicate bacteria within biofilms with higher efficiency than certain important antibiotics, making them interesting candidates for the treatment of biofilms.

Microbial biofilms pose a challenge in clinical and industrial settings where the need for sterility is paramount. Bacteria within biofilms are more resistant to antibiotics and disinfectants than individual cells in suspension (6, 25). Several mechanisms can account for the increased antibiotic resistance in biofilms, including the physical barrier formed by exopolymeric substances (14), a proportion of dormant bacteria that are inert toward antibiotics (15), and resistance genes that are uniquely expressed in biofilms (17, 19, 16, 27). Together, these bacterial features that create resistance to antibiotics drive the need for novel strategies that will effectively kill bacterial biofilms.

Plant essential oils have been used for hundreds of years as natural medicines to combat a multitude of pathogens, including bacteria, fungi, and viruses (10). Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lysis, leakage of cell contents, and inhibition of proton motive force (4). In addition, there is evidence that they effectively kill bacteria without promoting the acquisition of resistance (1, 22). Finally, many essential oils are relatively easy to obtain, have low mammalian toxicity, and degrade quickly in water and soil, making them relatively environmentally friendly (11).

Here, we probed the ability of selected essential oils to kill biofilms formed by *Pseudomonas aeruginosa* (PAO1), *Pseudomonas putida* (KT2440), and *Staphylococcus aureus* SC-01. *P. aeruginosa* is a Gram-negative bacterium found in soil, water, and animals, but it is also an opportunistic pathogen in humans. It can infect the pulmonary and urinary tracts, wounds, and burns and cause devastating medical complications by forming biofilms on medical devices, such as catheters. The biofilms formed by *P. aeruginosa* allow this pathogen to evade treatment with antibiotics and cause persistent, sometimes deadly, infections. The closely related species *Pseudomonas putida* can also form biofilms, but it is not a pathogen. Usually, *P. putida* is found in the environment, especially in soil, in freshwater, and on the roots of plants. The Gram-positive species *S. aureus* can exist both as a commensal and as a pathogen. As a pathogen, this bacterium is responsible for a broad range of maladies, from superficial skin infections to serious systemic infections. Treatment of *S. aureus* is complicated by antibiotic resistance, which is especially problematic in multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA).

Essential extracts from the bark of plants in the genus *Cinnamomum* have antibacterial activity toward a range of microbes, including *P. aeruginosa* (2, 21, 24). In previous studies, the effect of *Cinnamomum* extract on *P. aeruginosa* was tested against individual bacteria in solution. Here, we asked if this potent antimicrobial would also be effective against this bacterium within a biofilm.

To address this question, *P. aeruginosa* biofilms were grown on the air-liquid interface of a microscope slide, which was halfway submerged in Mueller-Hinton broth (MHB) containing PAO1 at an optical density at 600 nm (OD_{600}) of 0.0025. After 24 h of growth at room temperature, biofilms were washed with H_2O and then challenged with cation-adjusted MHB containing 0.2% or 0.1% (vol/vol) cassia oil (*Cinnamomum aromaticum*, 100% pure; Aura Cacia) or 3 μg ml^{-1} colistin. In a separate assay, using the CLSI broth microdilution method modified with a 2-hour challenge period (7), 0.2% (vol/vol) cassia oil and 3 μg/ml colistin were determined to be the lowest concentrations of these chemicals required to eradicate *P. aeruginosa* in solution (Table 1). In the case of cassia oil, 0.1% (vol/vol) Tween 80 was added to mix the oil with the medium (5). At this concentration, Tween 80 did not affect the growth or viability of planktonic cells or cells in a biofilm (data not shown). After 2 h, the treated biofilms were rinsed with H_2O, stained with LIVE/DEAD BacLight (Invitrogen), and imaged by wide-field fluorescence microscopy. BacLight uses a combination of two nucleic acid dyes: SYTO9, a membrane-permeable green dye that labels both viable and dead cells, and propidium iodide, a membrane-impermeative red dye that labels only membrane-compromised cells and eliminates the green SYTO9 signal. Planktonic cells (final OD_{600} = 0.25) were challenged with the same concentration of cassia oil or colistin used against the biofilms for 2 h and then placed into a glass-bottom 96-well plate for imaging.

Our results show that the MIC of colistin (3 μg ml^{-1}) needed to eradicate planktonic cells was not effective against cells within a biofilm, since a large fraction of the cells remained stained in green (Fig. 1, top right). In contrast, the MIC of cassia oil against planktonic cells (0.2%) (Table 1) was also sufficient to kill the vast majority of *P. aeruginosa* cells within a biofilm (Fig. 1, middle), suggesting that these cells were not protected from cassia oil. A slightly lower concentration of the essential oil (0.1%) did not kill bacteria in solution or in biofilms (Fig. 1, bottom).

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TABLE 1 MICs from broth microdilution assay[a]

Compound	MIC for:	
	P. aeruginosa PAO1	*P. putida* KT2440
Colistin	3.0 μg ml^{-1}	Not tested
Cassia oil	0.2% (vol/vol)	0.2%
Clove oil	>5%	>5%
Lavender oil	>5%	>5%
Peru balsam oil	2.5%	2.5%
Red thyme oil	>5%	2.1 ± 0.4%
Tea tree oil	5%	2.5%

[a] MICs of colistin and essential oils were determined by the standard broth microdilution assay. The data are averages; each experiment was performed in triplicate. The highest concentration of each essential oil tested was 5% (vol/vol). MICs of oils that did not show antimicrobial activity in the range tested are listed as ">5%." Standard errors are reported unless the results for all three trials were identical.

Are other antimicrobial essential oils as effective as cassia oil in killing *Pseudomonas* biofilms? To address this question, we screened for oils that can kill *P. aeruginosa* PAO1 in a disc diffusion assay using MHB agar according to the Clinical Laboratory and Standards Institute protocol (8). The essential oils were supplied by Aura Cacia and New Directions Aromatics and were described as 100% pure. Twenty microliters of each oil was spotted undiluted onto filter paper discs created from 3 layers of Whatman filter paper. Our data revealed that the following oils were effective in killing *P. aeruginosa*: cassia, clove (*Syzygium aromaticum*), Peru balsam (*Myroxylon balsamum*), red thyme (*Thymus vulgaris*), and tea tree (*Melaleuca alternifolia*) oils (Fig. 2). To account for the possibility that the oils penetrate the agar to different degrees, resulting in what falsely appears to be a reduced antimicrobial effect, any oil that produced a visible zone of inhibition was considered for subsequent experiments.

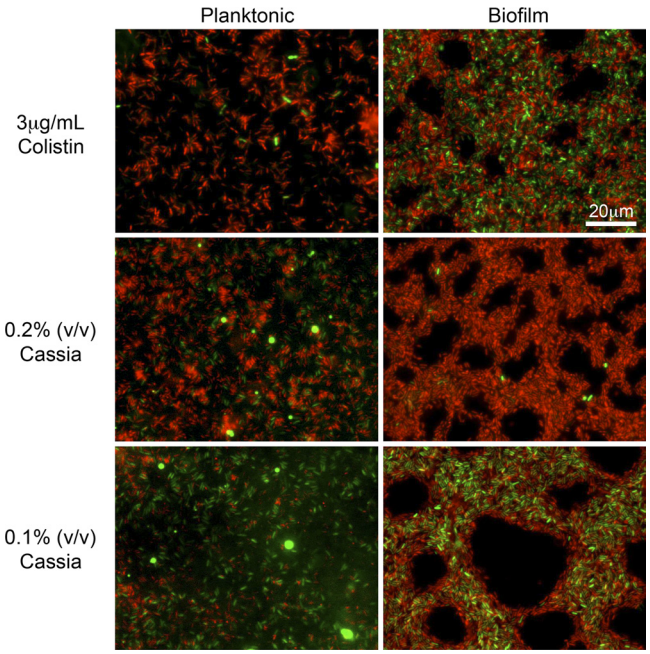

FIG 1 Cassia oil kills planktonic bacteria and biofilms with comparable efficiency. Cells were exposed to colistin or cassia oil for 2 h and then stained with a LIVE/DEAD stain to determine viabililty. Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

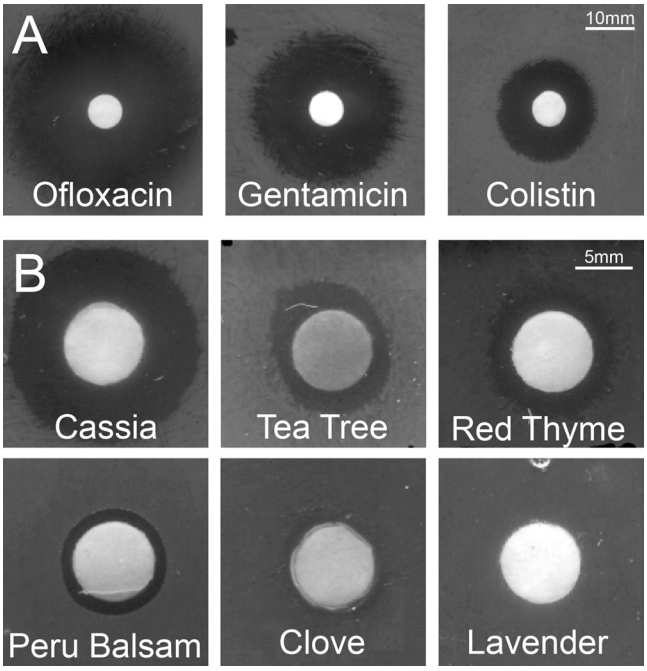

FIG 2 Disc diffusion assay identifies essential oils with antimicrobial activity. Antibiotics at a concentration of 20 mg ml^{-1} (A) and pure essential oils (B) were tested against *P. aeruginosa* PAO1. The substances that produced a zone of inhibition were further analyzed; lavender oil served as a negative control.

In the next step, we explored whether the oils that were active in the disc diffusion assay are also effective in killing biofilms. To address this point, we determined two parameters for individual oils: the MIC required to kill planktonic cells and the minimal biofilm eradication concentration (MBEC). Biofilms were grown on an MBEC device (Innovotech Inc., Edmonton, Canada), a modified microtiter plate that contains 96 polystyrene pegs attached to the lid (6). The pegs were immersed in MHB containing 10^6 cells ml^{-1} with shaking at 37°C and 30°C for *P. aeruginosa* PAO1 and *P. putida* KT2440, respectively. After 24 h, the biofilms that grew on the pegs were rinsed and subjected to a 1:1 serial dilution of antibiotics and essential oils in cation-adjusted MHB as indicated in Fig. 3; the medium used to dilute the oils was supplemented with 0.1% Tween. The volume of the challenge medium was 200 μl, and the highest concentrations of antibiotics and essential oils tested were 100 μg ml^{-1} and 5%, respectively. Ampicillin and lavender served as negative controls for antibiotics and essential oils, respectively. After 2 h of incubation, the pegs were washed, immersed in 150 μl fresh MHB, and sonicated for 10 min in a Branson 2510 sonicator (40 kHz) to release and dissociate the peg-associated biofilms. The average number of cells on each peg was determined by breaking the pegs off the lid and sonicating them individually in microcentrifuge tubes containing 200 μl of phosphate-buffered saline (PBS). The resulting solution was serially diluted and plated to determine CFU. The CFU counts revealed that the average numbers of cells per peg were 3×10^7 for PAO1 and 4×10^6 for KT2440. To obtain the MIC, the same number of planktonic cells was added per well for challenge with antibiotics or essential oils. After 2 h, 20 μl from each well was

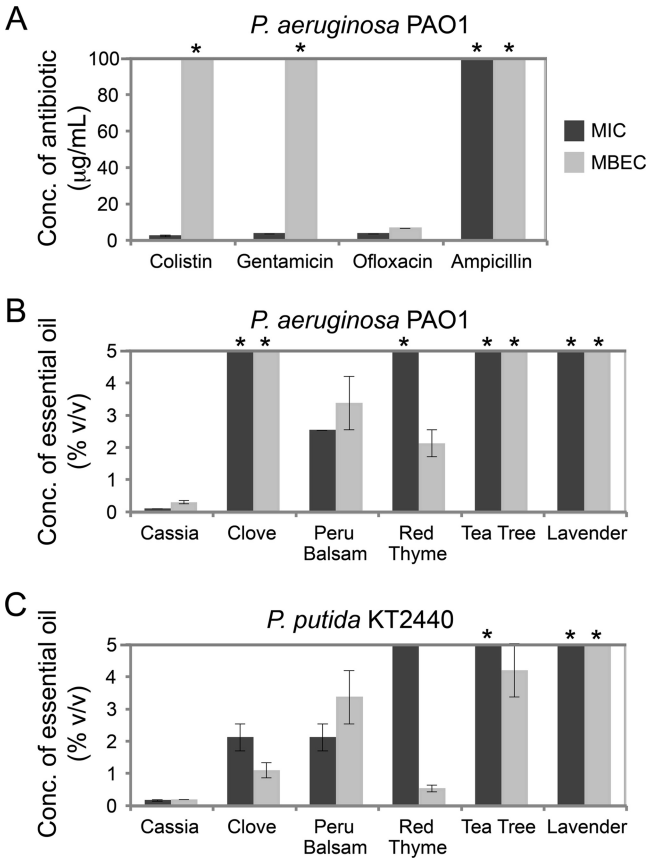

FIG 3 Activities of selected antibiotics and antimicrobial essential oils against *P. aeruginosa* PAO1 (A, B) and *P. putida* KT2440 (C). The MIC and MBEC of various substances were determined by challenging bacteria that were planktonic or within biofilms, respectively. Asterisks represent data that extend beyond the plot range, indicating that no killing was observed at the tested concentrations. Each experiment was performed in triplicate, and the error bars represent standard errors.

added to fresh MHB. After overnight incubation, the lowest concentration of each chemical that prevented survival of the biofilm and planktonic cells was determined. The experiments were performed in triplicate, and the average MICs or MBECs were determined.

Figure 3A and B show the MIC and MBEC of each substance tested. Only one antibiotic, ofloxacin, was able to eradicate planktonic and biofilm bacteria with almost equal efficiencies. The other antibiotics, colistin and gentamicin, were not effective in killing biofilms, even at concentrations 10-fold higher than the MIC. In contrast, cassia and Peru balsam essential oils were effective against biofilms and planktonic bacteria at nearly equal concentrations. This observation confirms the result in Fig. 1 for cassia oil, where little difference between the MIC and MBEC was observed. Interestingly, red thyme oil was effective against biofilms at a concentration of ~2% but was unable to kill planktonic cells at any of the concentrations tested. This suggests that thyme oil is more effective against biofilms than it is against bacteria in solution. To determine statistical significance, we performed a one-sample *t* test to compare the biofilm population to the mean of the planktonic population. Since the planktonic population for red thyme was not killed by the highest concentration tested, we used the maximum value (5%) as the population mean to see if significance could be detected at this level. Indeed, the differences between the planktonic and biofilm populations for both PAO1 and KT2440 were significant ($P < 0.05$), indicating that red thyme oil is more effective against biofilms than planktonic cells. We conclude that the essential oils tested here can act against biofilms more effectively than the tested antibiotics.

To test for potential strain-specific effects of the essential oils, we assessed their effect on a close relative, *P. putida* (KT2440) (Fig. 3C). Our data illustrate that *P. putida* is more sensitive than *P. aeruginosa* to clove, red thyme, and tea tree oils (Fig. 3). This effect is especially evident for clove oil, which did not eliminate PAO1 but was potent against KT2440 biofilms and planktonic cells (Fig. 3). Using one-sample *t* tests to compare the *P. putida* and *P. aeruginosa* data for clove oil (assuming that the mean for the *P. aeruginosa* samples is 5%), we found that the effect of clove oil on *P. putida* is significantly different from that on *P. aeruginosa* ($P < 0.05$). Additionally, red thyme oil was effective against planktonic bacteria at 5%, the highest concentration tested, and tea tree oil was effective against biofilm cells. We are unable to calculate statistical significance in these cases because *P. aeruginosa* survived at the highest concentration tested. The differences between the two *Pseudomonas* strains indicate species-specific activity of the oils and suggest that specific mechanisms of resistance to the oils may be at work. For example, since certain essential oils appear to work on the cell wall or cell membrane, it is possible that the composition of these cellular components is key to determining susceptibility to essential oils. The species-specific activity of the oils suggests that tailored combinations to target a range of different microbes may be effective against multispecies biofilms.

One similarity between the *P. aeruginosa* and *P. putida* data is that red thyme oil was more effective against biofilm cells than their planktonic counterparts. The same is true for tea tree oil against *P. putida*, which was ineffective at concentrations of 5% or less against planktonic bacteria but was effective against biofilm bacteria at a concentration of ~4%. In these cases, being inside a biofilm becomes a disadvantage for the bacteria, as it renders them more susceptible to the activity of these particular essential oils. It is possible that the extracellular matrix of the biofilm adsorbs the active components and increases their local concentration. Another possibility is that the cell membrane or cell wall in biofilm cells is different from that in planktonic cells due to differential gene expression in the two cell types.

It should be noted that the data obtained using the MBEC device are reproducible with a different assay where biofilms are grown in the wells of a 96-well microtiter plate (9) instead of on polystyrene pegs (Fig. 4). In this assay, the protocol is the same as that for the MBEC device except that that the plates are not shaken during incubation, allowing the biofilms to grow on the sides of the wells at the air-liquid interface. Additionally, the biofilms are formed in TB (10% tryptone, 5% NaCl) as opposed to MHB. As shown in Fig. 4, we compared the susceptibility of biofilms that were grown with both approaches. The biofilms grown with the two different methods contained comparable numbers of cells (2×10^7 CFU/well versus 3×10^7 CFU/peg). The MBEC values obtained for each method were the same for all substances except for ofloxacin. It is unclear why cells grown on the MBEC device were more susceptible to ofloxacin than cells grown in 96-well plates, especially considering that the other substances tested did not show significant variation in efficacy. However, it is possible

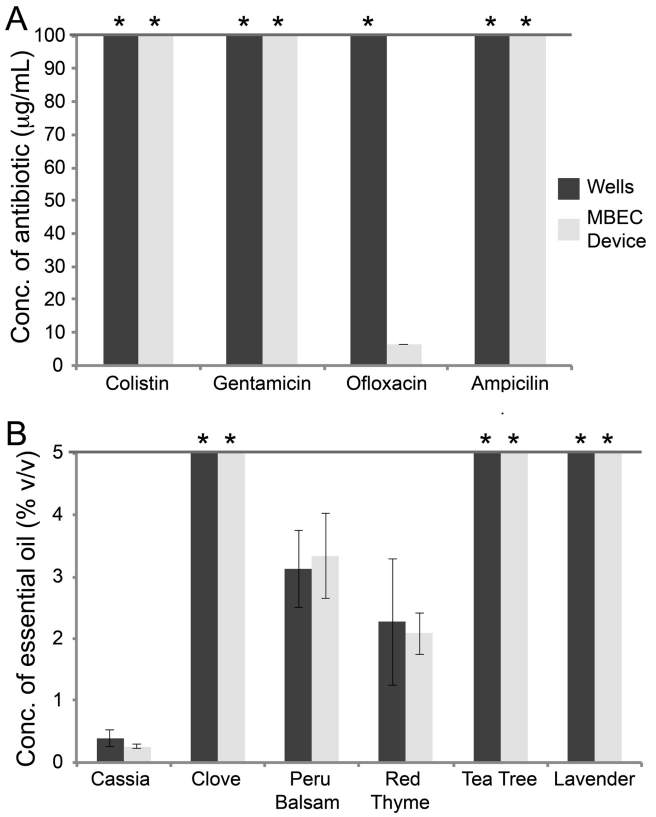

FIG 4 Comparing two methods of biofilm cultivation for antibiotic and essential oil testing. *P. aeruginosa* biofilms were grown either on the sides of wells in a 96-well plate or on the pegs of an MBEC device, and their sensitivities toward antibiotics and essential oils were determined.

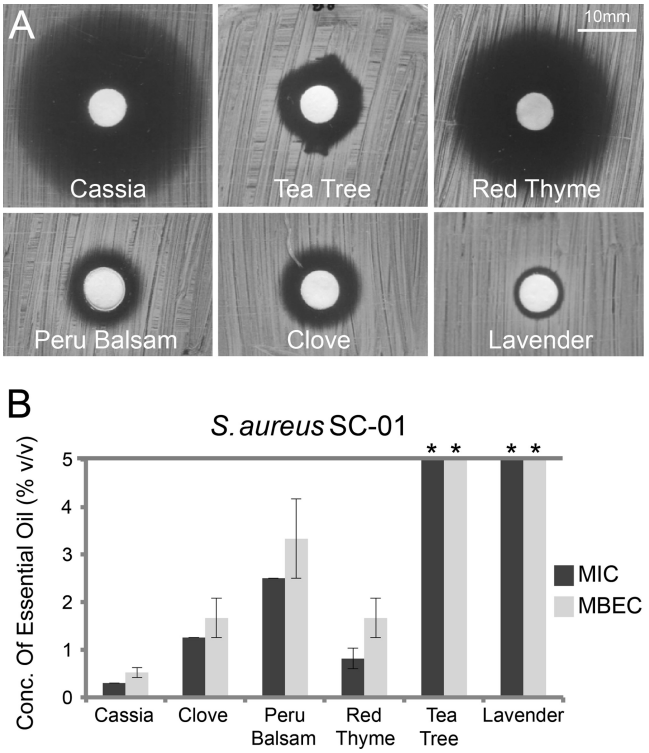

FIG 5 Susceptibility of *S. aureus* SC-01 to essential oils. (A) A disc diffusion assay reveals that SC-01 is sensitive to various essential oils. (B) The MIC and MBEC of essential oils were determined by challenging planktonic cells and biofilms, respectively. Asterisks represent data that extend beyond the plot range, indicating that no killing was observed at the tested concentrations. Each experiment was performed in triplicate, and the error bars represent errors.

that the difference in the medium or structure of the biofilms caused the increased susceptibility of biofilms grown on the MBEC device to ofloxacin.

After testing two closely related Gram-negative bacteria, we studied the effect of essential oils against the Gram-positive bacterium *S. aureus* (Fig. 5). Our goal was to determine whether the oils discriminate between Gram-positive and Gram-negative bacteria. The strain used in this study, SC-01, is a biofilm-forming, oxacillin- and methicillin-resistant clinical isolate (26). Certain essential oils, such as tea tree, thyme, and peppermint, are effective against planktonic (5, 18, 20) and biofilm (3, 12) MRSA. However, to our knowledge, essential oils from cassia, red thyme, and clove have not been tested against MRSA biofilms of any strain. Moreover, the strain used in this study (SC-01) has not been challenged with essential oils in previous work. First, we performed a disc diffusion assay to determine if the oils that are effective against *Pseudomonas* also work against *S. aureus*. Indeed, all of the oils tested, including lavender oil, showed a zone of inhibition (Fig. 5A). Next, we tested the essential oils against biofilms formed on the pegs of the MBEC device. The protocol is the same as described above, and the average number of cells per peg was 1.5×10^5. The results show that the biofilms were killed by the same or similar concentrations of cassia, Peru balsam, and red thyme oils as were effective against *P. aeruginosa* (Fig. 5B). Notably, this isolate is resistant to oxacillin and methicillin yet is killed effectively by four essential oils tested in this assay.

After determining that essential oils are effective against biofilms, we tested individual components of the essential oils for antimicrobial efficacy. We assessed the molecules cinnamaldehyde, eugenol, and linalool (from cassia, clove, and lavender oils, respectively) (13, 23) for their effect against *P. aeruginosa* planktonic and biofilm cells. All three components were obtained from Sigma-Aldrich. The protocol used in this assay is identical to that used for testing whole essential oils, including the use of Tween 80 in the medium to suspend the components, which have a low solubility in water. Table 2 summarizes the data, which indicate that cinnamaldehyde is as effective as the complex cassia oil. Additionally, whereas clove oil was not effective in killing *P. aeruginosa* biofilms in 5% (vol/vol) solutions, eugenol was effective at 3.3%. The finding that single essential oil components are effective at eradicating bacterial biofilms is promising, as it may allow for

TABLE 2 MIC and MBEC of essential oil components against PAO1[a]

Compound	MIC (% [vol/vol])	MBEC (% [vol/vol])
Cinnamaldehyde	0.1	0.2
Eugenol	>5	3.3 ± 0.8
Linalool	>5	>5

[a] Each experiment was performed in triplicate, and the results shown are the averages from the three trials. The highest concentration of each component tested was 5% (vol/vol). The MICs and MBECs of components that did not show antimicrobial activity in the range tested are listed as ">5%." Standard errors are reported unless the results for all three trials were identical.

the dissection of their mechanisms of action as well as inspire the molecular design of new antimicrobial components.

In summary, we demonstrate here that cassia, Peru balsam, and red thyme essential oils are more effective in eradicating *Pseudomonas* and *S. aureus* biofilms than selected important antibiotics, making them interesting candidates for the treatment of biofilms. Important future goals include identifying further active antimicrobial components within the oils, as well as the molecular mechanisms by which these components so effectively breach the biofilm barrier. In this study, we sampled only a small number of different oils, but a plethora of other oils is available in nature, bearing enormous potential for the discovery of alternatives to antibiotics.

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